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A solid-phase assay for quantitative analysis of sulfated glycosaminoglycans at the nanogram level. Application to tissue samples

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Abstract

A sensitive and accurate solid-phase methodology for the quantitative analysis of glycosaminoglycans is described. Chondroitin-4-sulfate (CSA) was labelled with biotin hydrazide after the reaction of its carboxyl groups with it in the presence of carbodiimide. Polystyrene plates modified with sequential reaction with glutaraldehyde (GH) and spermine to possess amino groups were used to immobilize electrostatically the biotin labelled CSA. Exogenously added sulfated glycosaminoglycans (GAGS) [variously sulfated chondroitin sulfates and heparan sulfate (HS)] were found to compete to this immobilization in a concentration dependent mode, within a concentration range from 10 up to 300 ng/ml. Glycosaminoglycan-derived oligosaccharides competed to a degree similar to that of intact molecules. Hyaluronan (HA) and keratan sulfate (KS) did not compete the immobilization. The procedure was applied for the rapid and reproducible determination of the sulfated glycosaminoglycans in proteinase digests of small tissue samples or cell cultures with high sensitivity and accuracy.

Keywords: Sulfated glycosaminoglycans; Solid-phase assay

1. Introduction

Glycosaminoglycans (GAGs) are a class of biological macromolecules of varying size, involved in a wide range of different biological processes. They are mainly present in the extracellular matrix of cartilage and other connective tissues (skin, tendon, bone, aorta, etc.). Tissues other than connective (brain, kidney, colon, etc.) are also found to contain GAGs, but in minor amounts (for reviews, see [1–3]).

GAGs are linear polymers of a disaccharide repeating unit formed by a hexuronic acid and a 2-amino-2-deoxy-hexose (hexosamine) residue [1]. Glucuronic acid is present in hyaluronan (HA) and chondroitin sulfate, while both glucuronic and iduronic acids are found in dermatan sulfate, heparan sulfate (HS) and heparin. Glucosamine is present in HA, HS and heparin, and galactosamine is present in chondroitin sulfate and

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dermatan sulfate. Keratan sulfate (KS) is the only GAG that contains galactose instead of glucuronic acid. An important feature of the GAG structure is the presence of a sulfate ester group in the hexosamine residues, in the case of chondroitin sulfate, dermatan sulfate, heparin, HS and KS, that makes the GAG molecule of high negative charge. In addition, an extra sulfate ester group is also found in some iduronic acid residues of HS and heparin. Hexosamine is usually *N*-acetylated, but in the case of heparin and HS some residues are also *N*-sulfated [1,4]. Additional sulfate esterification of GAGs is also observed in many cases, but in minor percentages, with the exception of invertebrate GAGs, where their oversulphation seems to be the physiological state.

GAG analysis is performed by a variety of assays, involving either chemical reactions of the carbohydrate constituents, especially in preparations rich in GAGs [5–8] or chromogenic reactions of the anionic groups [9–12]. For the analysis of small amounts of GAGs, such as the biopsy specimens or cell cultures, more complex techniques, involving HPLC and HPCE after the degradation of GAGs by specific enzymes (for reviews see [13,14]), have been established. Other specific procedures have also been established for the determination of minor amounts of heparin, based on its anticlotting activity and for HA, based on its ability to bind cartilage link protein [15] or aggrecan G1 domain [16].

The quantitation of GAGs is of great importance, especially when differences in the composition of a tissue sample or of a cell culture have to be detected for diagnostic purposes, such as cancer [17–24], inflammatory disease [25–27] and ageing [28–31]. In such cases, due to sample limitation, development of highly sensitive techniques with no further specific handling and/or sophisticated requirements is of great importance. The aim of the present work was, therefore, the development of a sensitive and accurate quantitative assay for the determination of minor amounts of sulfated GAGs, by a solid-phase assay which may be very useful for routine analytical purposes.

2. Experimental

2.1. Chemicals and biological material

Polystyrene plates were obtained from Kima (Italy). Glutaraldehyde (GH), 70% (w/w) was purchased from Serva (Germany). Biotin hydrazide, HS and dermatan sulfate (chondroitin sulfate B, CSB) from bovine intestinal mucosa, chondroitin sulfate type II (equimolar mixture of chondroitin sulfate from shark and whale cartilage), heparin, testicular hyaluronidase, avidin conjugated with peroxidase, Sephadex G-100 and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) were obtained from Sigma Chemical (USA). Chondroitin-4-sulfate (chondroitin sulfate A, CSA) from pig laryngeal cartilage and sheep nasal cartilage, HA from rooster comb, KS from sheep nasal cartilage and chondroitin sulfate E from squid cranial cartilage were isolated as described previously [28,29,32,33]. All other chemicals used were of the best available grade.

2.2. Analytical methods

Quantitation of the various GAGs in solution was performed with either the borate carbazole [6] or the dimethylmethylene blue (DMMB) [10,11] assay. The concentration of commercial preparations of GAGs was calculated from the weight of GAG dissolved in known amount of 2 × distilled water.

2.3. Enzymic digestions and molecular size determination

Limited digestion of CSA with testicular hyaluronidase was performed in sodium chloride (0.15 M)-sodium acetate (pH 5.3; 0.1 M) at 37°C for 4 h, using 0.1–1 unit of the enzyme per 1 mg of substrate and 1 ml of buffer. The enzymic reaction was terminated by boiling the solutions for 5 min. The molecular size of the products was determined following chromatography on a Sephadex G-100 (120 × 0.55 cm i.d.), eluted with sodium chloride (0.15 M)-sodium phosphate (pH 7.3; 0.1 M), which was calibrated with HA-derived oligosaccharides of known molecular mass [34,35].

2.4. Biotinylation of CSA

Biotinylation of CSA from pig laryngeal cartilage was performed essentially as described by Yang et al. [36] and Frost and Stern [37]. In brief, 1 ml of CSA (2 mg/ml) was mixed with 21 μl of biotin hydrazide (5 mg/ml) and 15 μl of EDAC (10 mg/ml), all being dissolved in sodium borate (pH 5.2; 0.1 M). The mixture was left for reaction at 23°C for 16 h with gentle agitation, and then was dialysed at 23°C against sodium acetate (pH 4.0; 0.5 M) for 4 h and against sodium chloride (0.14 M)-sodium phosphate (PBS) (pH 7.2; 0.01 M) for 16 h. The dialysate was chromatographed on a Sephadex G-100 column eluted with PBS (pH 7.2) and the fractions containing CSA were collected, pooled, made up to 30% (v/v) in glycerol and stored at −20°C.

2.5. Immobilization of biotinylated CSA

The procedure applied for the immobilization of biotinylated CSA (b-CSA) was based on its negative charges. In polystyrene plate wells, positive charges were first introduced by the sequential reaction of glutaraldehyde and spermine, almost exactly as described previously by our laboratory [38]. In brief, 100 μl of 1.25 mM GH in sodium phosphate (pH 5.0; 0.1 M) was added to the wells and incubated for 16 h at 23°C. The wells were washed twice with the same buffer and 100 μ1 of 50 gM spermine in sodium phosphate (pH 9.0; 0.1 M) were added and incubated for 4 h at 37°C. The wells were then washed twice with sodium chloride (0.15 M). Any remaining non-blocked sites were covered using 1% (w/v) bovine serum albumin — 0.1% (w/v) Tween 20 in PBS (pH 4.3) (PBS-T) at 37°C for 1 h. The wells were washed four times with PBS-T, and in the modified wells, various concentrations (1 ng/ml up to 10 μg/ml in 100 μl of PBS-T) of b-CSA were added and incubated at 37°C for 1 h. Following four washings with PBS-T, 100 μl of avidin–peroxidase conjugate diluted in PBS-T (1:10 000) was added to the wells and the plates were incubated at 37°C for 1 h. At the end, the wells were washed four times with PBS-T and 100 μl of o-phenylenediamine solution [2 mg/ml in sodium citrate (pH 5.5; 0.05 M), containing 10 mM hydrogen peroxide] were added to each well, the plates were incubated for 30 min at 25°C and the reaction was stopped by the addition of 100 μl of sulphuric acid (1 M). The color developed was measured by a microplate reader at 492 nm.

2.6. Inhibition of the immobilization of b-CSA by various GAGs

The immobilization of b-CSA onto the modified plate wells is mainly due to electrostatic forces and therefore other GAGs would inhibit it. The extent of this inhibition was studied after the simultaneous addition of b-CSA and varying concentrations of the GAGs in 100 μl of PBS-T. The GAGs used were: CSA; dermatan sulfate (CSB); HA; HS; KS; oversulfated chondroitin (CSE); and chondroitin sulfate oligosaccharides of variable molecular size. The concentration of b-CSA was 0.2 μg/ml and that of the polyanions ranged from 0.1 μg/ml up to 20 μg/ml. The extent of the inhibition of the immobilization of b-CSA caused by each of the polyanions was calculated from reference wells where b-CSA alone was used.

3. Results and discussion

3.1. Optimisation of the conditions for the immobilization of b-CSA

CSA was biotinylated using such concentrations of biotin hydrazide and EDAC to result in a sparse substitution of carboxyl groups with biotin (approximately one biotin per decasaccharide) [37]. This ensured that biotinylation of CSA do not affect significantly its charge density and chemical structure. The optimum conditions for maximum binding of b-CSA to plate wells modified to contain amino groups were also studied. It was found that almost all conditions examined were the same with those previously described for the electrostatic immobilization of aggrecan with the exception that optimum pH of the PBS-T buffer was found to be 4.3 instead of 4.8 used for aggrecan [38]. Lower pH values abolished the immobilization, as it was expected, be-

cause of less anionic charges present on b-CSA. Using higher pH values high absorbance backgrounds (blank values) were obtained, and this may well be attributed to the very strong positive charge of avidin. Once the conditions were established, b-CSA was added to the wells at various concentrations, left to be immobilized to them and detected by avidin-peroxidase conjugates, using o-phenylenediamine as coloring reagent (Fig. 1). The reaction proceeded rapidly as the concentration of b-CSA increased, reaching a maximum value at a concentration value of ≈ 2 μg/ml, whereafter remained almost constant. Using a concentration as small as 200 ng/ml, the color produced gave an absorbance value of higher than 1.0, in accordance to the high sensitivity expected for the biotin-avidin system used. Non-biotinylated CSA showed no ability to interact with avidin and, therefore, the absorbance values obtained with the immobilized b-CSA were exclusively due to the presence of biotin covalently bound on CSA (Fig. 1).

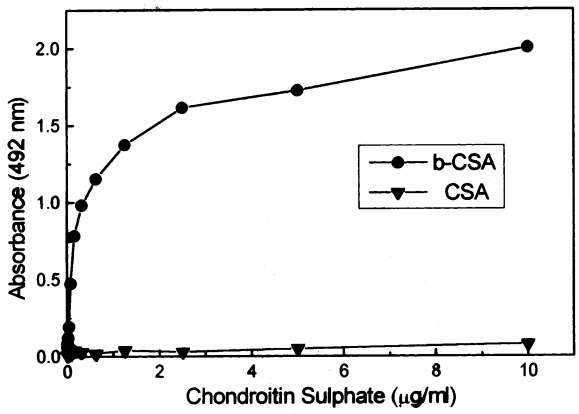

Fig. 1. Immobilization of biotinylated chondroitin-4-sulfate (b-CSA) on spermine coated 96-well microplate: Various concentrations of b-CSA were added to the wells and left to interact electrostatically with bound spermine. The amount of immobilized b-CSA was measured after the addition of avidin-peroxidase conjugate and finally by the addition of hydrogen peroxide and o-phenylenediamine. For comparison, the curve obtained using non-biotinylated CSA is included.

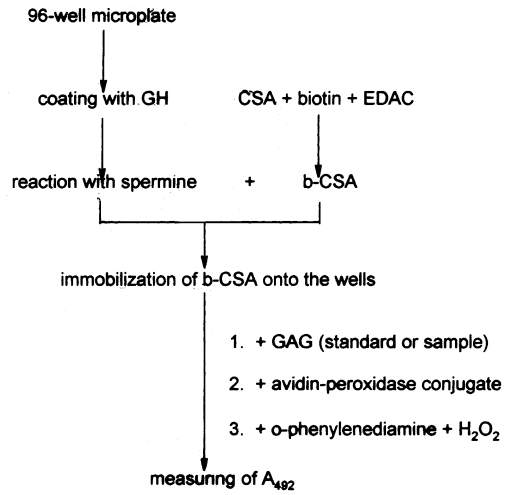

Fig. 2. Flow diagram showing the strategy followed for immobilization and determination of sulfated glycosaminoglycans.

3.2. Quantitative analysis of GAGs

A representative flow diagram followed for immobilization and determination of sulfated GAGs is presented in Fig. 2. The analysis of the various GAGs was based on their ability to compete with b-CSA for the spermine amino groups. This ability might be influenced by the charge density, the size and the structure of each GAG, and so, all parameters were examined.

The effect of the charge density of a GAG in inhibiting b-CSA interaction with spermine is shown in Fig. 3A. As shown therein, the competition effect of each GAG was directly related to its charge density. HS and CSE, which have more than two negative charges per disaccharide unit, i.e. one carboxyl group and one or two esterified sulfates, competed with b-CSA for spermine at concentrations lower than those of CSA. The latter contains two negative charges per disaccharide unit, and, therefore, may be considered as a reference molecule for this assay. It should be noticed here, that CSA gave a 50% concentration inhibition value which was the same with that used for immobilizing b-CSA. This verified the hypothesis that sparse biotinylation of CSA under the applied conditions did not have any effect on both its chemical structure and charge density. CSB, another GAG which bears two negative charges per disaccharide unit, showed exactly the same competition curve as CSA (Fig. 3A). Since

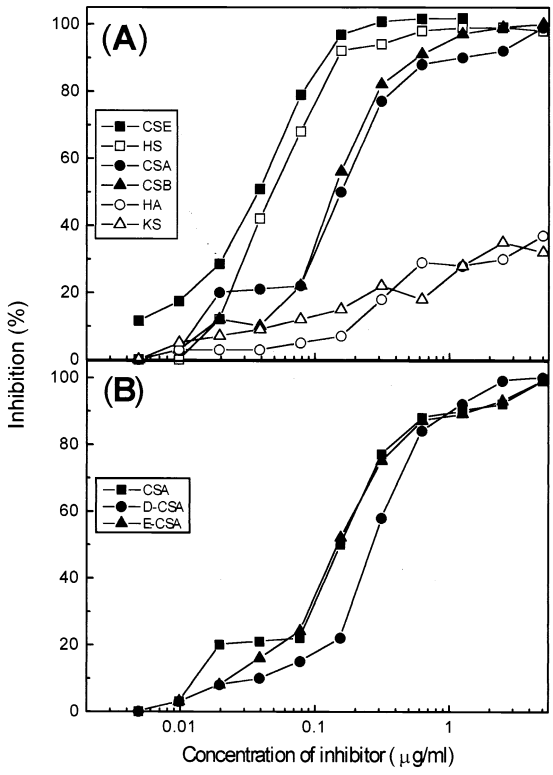

Fig. 3. Inhibition profiles of the b-CSA immobilization onto spermine coated wells by various GAGs (A) and CSA-derived oligosaccharides (B). b-CSA (200 ng/ml) and various concentrations of GAGs or oligosaccharides were added to the wells and left to interact with the well-bound spermine. The inhibition values were estimated from reference wells, where the competitor was omitted. D-CSA: CSA-derived decasaccharide; E-CSA: CSA-derived eicosasaccharide.

CSB differs from CSA in having a large part of its uronic acid residues as iduronic acid, this finding suggested that C-5 epimerization of glucuronate did not influence the competition curve as compared to that obtained with glucuronic acid-containing CSA. HA, bearing only one carboxyl group per disaccharide unit, as well as KS, which lacks of uronic acid and contains only one sulfate per disaccharide unit, showed very low inhibition values and only very high concentrations are necessary for competing with b-CSA (Fig. 3A). However, no 50% inhibition profiles were obtained even at very high concentrations tested (up to 20 μg/ml).

The GAGs analysed were not only of different charge, but of different size as well and thus the obtained results might be owed to both parameters. Therefore, the effect of the size of a GAG sample in inhibiting b-CSA interaction with spermine was examined by using two CSA-derived oligosaccharide preparations, produced following limited treatment with hyaluronidase. Their average size, estimated from a calibrated column of Sephadex G-100, was found to be of eicosasaccharide and decasaccharide, respectively (not shown). As shown in Fig. 3B, the obtained inhibition curves were exactly the same as that of intact CSA. This finding suggested that the size of a GAG preparation did not affect its ability to inhibit b-CSA interaction with spermine and consequently the charge is considered as the only parameter affecting the interaction between b-CSA and spermine.

The linear part of the competition curves obtained reflected the concentration range of a GAG solution under which the method could be applied. HS and CSE could be quantitated accurately between 13 and 130 ng/ml and CSA and CSB between 90 and 300 ng/ml. From this range, the minimum amount of HS and CSE quantitated was 1.3 ng, and that of CSA and CSB 9 ng, since 100 μl have been used in this assay.

3.3. Applications

A commercial preparation of chondroitin sulfate (Sigma type II) and a preparation isolated from sheep nasal cartilage were dissolved in 2 × distilled water (1 mg/ml) and the solutions were analysed by the dye binding assay and the assay presented here. As reference substance CSA from pig laryngeal cartilage was used and the results from both assays were compared. The calculation of the concentration of the GAG solutions when the competitive assay was used was performed from the 50% inhibition value of the respective curve. The results obtained are presented in Table 1, and the shown values are the mean of four measurements. It can be observed that the assay presented here gave accurate and reproducible results, in good agreement with either simple weighing of the sample or analysing by the DMMB assay.

Table 1
Determination of the concentration of solutions of a commercial preparation of chondroitin sulfate and of chondroitin sulfate isolated from sheep nasal cartilage by the dimethylmethylene blue (DMMB) assay and by the competitive solid-phase assay[a]

Compound	DMMB (mg/ml)	Solid-phase assay (mg/ml)
Type II CS	1.032 ± 0.016	1.028 ± 0.023
Sheep CS	1.004 ± 0.005	1.001 ± 0.006

[a] As reference compound CSA from pig laryngeal cartilage was used. GAGs concentrations used were 1.0 mg/ml.

In addition to accuracy and reproducibility, the assay presented here is characterized by its high sensitivity, and therefore it can be applied for the determination of GAGs in very dilute solutions, such as those from cell cultures or from biopsy specimens. The assay is easily performed and very rapid, since more than 90 samples can be analysed within < 180 min. It should be mentioned here that the plates can be stored for at least 1 month at 4°C after the blocking step [38]. The inability for HA, a non-sulfated GAG, quantitation is not a disadvantage, since this polysaccharide can be quantitated from the difference of the result from the borate carbazole reaction and that of the competitive assay, or directly by another, plate-based assay [16]. Moreover, others, dye-based assays [9,11,12], have been applied for GAG quantitation, even if they can not measure HA. Similarly, the amounts of KS in a GAG mixture can be quantitated from the difference of the results of dye binding assay and that of the competitive assay.

Although highly sensitive HPLC and HPCE techniques have been described and successfully applied for GAGs analysis [13,14], the proposed solid-phase technique has the advantage of not requiring specific GAG degrading enzymes and furthermore it can be applied for determining the total amount of GAGs in biological samples.

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